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# Inhibition of α-amylase and α-glucosidase enzymes by extracts of plants of the genus *Zanthoxylum*

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***Abstract*****—** *Diabetes mellitus type 2 represents 90% of all cases of diabetes, being characterized by a series of metabolic dysfunctions, including hyperglycemia. The literature points to a-amylase and a--glucosidase as potential therapeutic targets for the development of medicines to treat postprandial hyperglycemia. Among the plants of the semiarid region of Bahia, species of the genus Zanthoxylum have phytochemicals with pharmacological potential not yet explored. Therefore, the present work aims to evaluate the in vitro effect of crude extracts of some plants of the genus Zanthoxylum on the enzymes α-amylase and α-glucosidase. A total of eleven stem, stem bark and leaf extracts of Z. monogynum and Z. rhoifolium were obtained by cold maceration in the hexane, ethyl acetate, ethanol and methanol solvents. The extracts were incubated with a-amylase or α-glucosidase, and residual enzyme activities were determined. Phenolics were quantified by the Folin-Ciocalteau method. All extracts reduced enzyme activity, especially the methanolic leaf extract of Z. monogynum reduced a-amylase activity (53.5%) and the stem bark extract of Z. rhoifolium of a-glucosidase (99.2%). In any experiment the inhibitory effect correlated with the concentration of phenolic compounds ($p > 0.05$). The half maximal inhibitory concentration ($IC_{50}$) values of the extracts in the case of a-amylase was 25.9 and 61.5 µg/mL, while in α-glucosidase $IC_{50}$ values were between 21.6 and 26.5 µg/mL. The results indicate that the extracts are potentially useful for the treatment of diabetes. Further phytochemical studies directed to the isolation of bioactive molecules and characterization of the mechanism are needed.*

***Keywords*****— diabetes tipo 2; α-amilase; α-glicosidase; Zanthoxylum.**

## I. INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder of multiple etiology, characterized by hyperglycemia resulting from insufficient insulin production, defect in its action, or both mechanisms (Garber et al., 2013; SBD, 2014). Thus, depending on its etiology, DM can be classified into type 1 DM, type 2 DM and gestational DM, among other specific types (Tonelli and Resende, 2013). In 2015, this syndrome affected about 415 million people worldwide (IDF, 2015).

Given the important role in the initial metabolism and intestinal absorption of carbohydrates, amylase αand glucosidase αhave been explored as therapeutic targets in the development of hypoglycemic drugs (Gamboa et al., 2017). Currently the commercially available enzyme inhibitors for the treatment of type 2 DM are acarbose, miglitol and voglibose. In general, these drugs act as reversible competitive inhibitors on the enzymes α-amylase and α-glucosidase. Although these drugs are well tolerated by patients, reports of gastrointestinal changes severely limit treatment adherence (Ali et al., 2006; Kim et al., 2011).

α-amylases (α-1,4-glycan-4-glycanhydrolases) are present in mammalian saliva and pancreatic juice and are classified as endoamylases, that is, they hydrolyze α-1,4-type glycosidic bonds inside starch. They are crucial in the early stages of the process of starch degradation to smaller subunits, the dextrins (Sales et al., 2012; Yang et al., 2012). Inturn, α-glucosidases (α-D-glycoside glycohydrolases) are classified as exoglycoside hydrolases because they cleave α-1,4 and α-1,6 glycosidic bonds from the non-reducing end of substrates, releasing α-D-glucose

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units. In men, they are attached to the surface of intestinal villi and actively participate in glucose absorption at these sites (Peng et al., 2016).

In recent years much effort has been directed to identifying natural sources of α-amylase and α-glucosidase inhibitor molecules in the attempt to develop safer and cheaper drugs for the treatment of postprandial hyperglycemia (Gonçalves et al., 2011). In this context, plants stand out for synthesizing a range of bioactive metabolites with enormous pharmacological potential (Gadelha et al., 2013).

Among the plants of the region, the family Rutaceae is one of the most important because it is widely distributed. This family has about 160 genera and 1900 species distributed worldwide (Groppo and Pirani, 2012). In Brazil, 33 genera and 193 different species have been cataloged in all regions (You et al., 2015). The genus *Zanthoxylum* has 250 species and has been used in folk medicine to treat a variety of diseases such as diabetes, tuberculosis, malaria, cardiovascular diseases, and has been used as pain killer and antiophidic (Karki et al., 2014; Aloke et al., 2014). Furthermore, phytochemical studies have identified alkaloids, flavonoids, coumarins, lignans and terpenes with antidiabetogenic, antinociceptive, antidiarrheal, antimalarial, antioxidant, immunomodulating, antimicrobial potential in this genus, among others (Krause et al., 2013; Krause, 2013; Fernandez et al., 2017).

Therefore, the present work aimed to evaluate the *in vitro* effect of extracts of *Zanthoxylum monogynum* and *Zanthoxylum rhofolium* on the activity of α-amylase and α-glucosidase. This investigation may contribute to the isolation of prototypes for new hypoglycemic drugs, as well as to promoting the rational and self-sustainable exploitation of the flora of the semiarid region of Bahia.

## II. MATERIAL AND METHODS

### *A. Materials*

Ethyl acetate, ethanol, hexane, methanol were purchased from Qhemis. Folin-Ciocalteau reagent, gallic acid, porcine pancreatic α-amylase type VI-A, α-glucosidase from Saccharomyces cerevisiae, ρ-nitrophenyl-α-D-glycopyranoside substrate (ρNPG), and sodium carbonate were purchased from Sigma Aldrich. The Amilase kit (K003) was kindly donated by Bioclin (Belo Horizonte, Minas Gerais).

### *B. Obtaining extracts*

The plants were collected at Brejo Novo Farm, in the city of Jequié, located in southwestern Bahia. Leaf and stem bark samples of *Z. monogynum* St.-Hil. and stem, stem bark, thick stem bark, and leaf samples of *Z.* rhoifolium Lam. were subjected to oven drying at 40°C. After trituration, they were thoroughly macerated with hexane, ethyl acetate, ethanol or methanol solvents (Cechinel and Yane, 1998). After removal of solvents in rotary evaporator with reduced pressure, the hexane, ethyl acetate, ethanolic and methanolic extracts were obtained. One sample of each plant was deposited at the Herbarium of the State University of Southwest Bahia, Jequié campus, with registration numbers HUESB 1348 (*Z. monogynum*) and 7737 (*Z. rhofolium*).

### *C. Determination of α-amylase activity*

The activity of α-amilase was determined using a commercial kit (Bioclin). The extracts (1 mg/mL) were preincubated with type VI-A porcine pancreatic α-amylase (0.1 mg/mL) in 50 mM phosphate buffer, pH 6.6, for 15 min at 37°C. Then an aliquot of the enzyme was incubated with starch (40 mg/mL) for 7.5 min at 37°C. After addition of iodine (4 mg/mL), the absorbance of the residual starch-iodine complex was measured at 660 nm in a spectrophotometer (GEHAKA UV340G). Acarbose (1 mg/mL) and solvent (5 µL) were used as positive and negative controls, respectively.

The inhibition of enzymatic activity was determined by the following formula:

$$Inibição\ (\%) = 100 - [(Abs\ A/Abs\ B)x100]$$

Where Abs A is the absorbance of the negative control at 660 nm and Abs B is the absorbance of the sample at 660 nm.

### *D. Determination of α-glucosidase activity*

The activity of α-glucosidase was determined using the modified Shinde method. The principle of the method is based on the hydrolysis of the chromogenic substrate ρ-nitrophenyl-α-D-glycopyranoside (ρNPG) to ρ-nitrophenol (ρNP) by α-glucosidase, producing coloration. α-glucosidase (*Saccharomyces cerevisiae*, 10 µg/mL) was incubated with the extracts (1 µg/mL) in 100 µL sodium phosphate buffer (50 mM), pH 6.8, for 15 min at 37°C. Then 40 µL of the substrate ρNPG (1 mM) was added. After a further 15 min at 37°C, the reaction was stopped by the addition of 100 µl sodium bicarbonate (10%). The absorbance of the product was measured at 405 nm in a microplate reader (Biotek ELx800®). Acarbose (1 mg/mL) and solvent (5 µL) were used as positive and negative controls, respectively.

The inhibition of enzymatic activity was determined by the following formula:

$$\text{Inhibition } (\%) = 100 - [(Abs_C/Abs_D)x100]$$

Where $Abs_C$ is the absorbance of the negative control at 405 nm and $Abs_{D\ is}$ the absorbance of the sample at 405 nm.

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*E. Determination of $IC_{50}$ values of extracts*

*Zanthoxylum* extracts that reduced the activity of α-amylase and α-glucosidase by more than 50% were selected for determination of the half maximal inhibitory concentration ($IC_{50}$). For the dose response curve, enzymes were preincubated with extracts at concentrations of 1.5 to 200 µg/mL. The subsequent steps were the same as the items for determining α-amylase and α-glucosidase activity. $IC_{50}$ values were determined by rectangular hyperbolic fitting of dose response curves using nonlinear regression in the GraphPad Prism 6.0 software.

*F. Quantification of total phenolics*

The concentration of total phenolic compounds present in *Zanthoxylum* extracts was determined by the Folin-Ciocalteau spectrophotometric assay described by Singleton[43]. Samples were diluted in their respective solvents to a final concentration of 1 mg/mL. An aliquot of 125 µL of the extract was mixed with 125 µL Folin-Ciocalteau reagent and 1 mL distilled water. After 3 min at room temperature, 125 µL of saturated of $Na_2CO_3$ solution was added and then incubated in water bath for 30 min at 37°C. At the end of incubation, the absorbance of the sample was measured spectrophotometrically at 750 nm in a quartz cuvette with 0.5 cm optical path. To quantify the total phenolics, a standard curve with 0.5, 5, 10, 15 and 25 µg gallic acid solution was used.

*G. Statistical analyses*

All analyses were performed in triplicate and the results were expressed as mean ± standard deviation. Data normality was checked using the D'Agostino-Pearson test ($p > 0.05$). The data were then compared by one-way ANOVA and Bonferroni post-hoc test for normally distributed data, and by the Kruskal-Wallis test and Dunn's post-hoc test for non-normally distributed data using the GraphPad Prism 6.0 software. The statistical significance level used was 5% ($p < 0.05$).

## III. RESULTS AND DISCUSSION

Initially, eleven *Zanthoxylum* extracts at 100 µg/mL were evaluated for their effect on the effect over α-amilase. The results of this screening demonstrated that only EMFZM and EMCCZR extracts were able to reduce enzyme activity by more than 50% (Table 1).

*Table 1. Inhibition of α-amilase by Zanthoxylum plant extract.*

| Extracts | Inibition (%)[a] |
|---|---|
| EMCCZM | 11,7 ± 0,5* |
| EMFZM | 53,5 ± 3,0 |
| EECGCZR | 21,3 ± 6,4 |
| EECZR | 11,7 ± 1,7* |
| EHCCZR | 36,7 ± 1,3 |
| EAECCZR | 52,1 ± 3,3 |
| EECCZR | 26,5 ± 1,5 |
| EMCCZR | 12,6 ± 0,6 |
| EHFZR | 22,5 ± 1,2 |
| EAEFZR | 10,8 ± 1,1* |
| EMFZR | 13,2 ± 0,3 |
| Acarbose | 87,6 ± 0,1 |

[a] The enzyme was preincubated with the extracts (100 µg / mL) and acarbose (100 µg / mL) for 15 min. * $p < 0.05$ when compared to acarbose according to Kruskal-Wallis followed by Dunn's posttest. EMCCZM: Methanolic extract of Zanthoxylum monogynum stem bark. EMFZM: Methanolic extract of Zanthoxylum monogynum leaves. EECGCZR: Ethanolic extract of thick stem bark Zanthoxylum rhoifolium. EECZR: Zanthoxylum rhoifolium ethanolic stem extract. EHCCZR: Zanthoxylum rhoifolium hexane stem bark extract. EAECCZR: Ethyl acetate extract of Zanthoxylum rhoifolium stem bark. EECCZR: Zanthoxylum rhoifolium ethanolic stem bark extract. EMCCZR: Zanthoxylum rhoifolium stem bark methanolic extract. EHFZR: Hexane Extract of Zanthoxylum rhoifolium Leaves. EAEFZR: Ethyl acetate extract of Zanthoxylum rhoifolium leaves. EMFZR: Methanolic extract of Zanthoxylum rhoifolium leaves.

As for *Z. monogynum*, the methanolic leaf extract (EMFZM) inhibited enzyme activity by 53.5%, while methanolic stem bark extract (EMCCZM) by only 11.7%.

In the case of the species *Z. rhoifolium*, extracts from different parts of the plant were evaluated. Crude thick stem bark extract (EECGCZR) caused a considerable

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inhibition of 21.3%, while ethanolic stem extract (EECZR) of this species showed an inhibition of only 11.7%. Among the crude extracts derived from stem bark, the ethyl acetate extract (EAECCZR) stood out by inhibiting α-amylase activity by 52.1%, while the hexane (EHCCZR), ethanolic (EECCZR) and methanolic (EMCCZR) extract reduced this activity by 36.7, 26.6 and 12.6%, respectively. Finally, the hexane extract (EHFZR), methanolic extract (EMFZR) and ethyl acetate extract (EAEFZR) of leaves reduced α-amylase activity by 22.5, 13.2 and 10.8%, respectively. These differences in activity within the same plant and between genera may be due to variation in concentration and type of secondary metabolites in different plant organs (Oliveira et al., 2016).

At the initial screening, EMFZM and EAECCZR extracts alone inhibited α-amylase by more than 50% and, therefore, curves with varying concentrations of these extracts (1.1 to 100 µg/mL) were plotted to determine $IC_{50}$ values (Figure 1). $IC_{50}$ values for EMFZM and EAECCZR extracts were 25.9 and 61.6 µg/mL, respectively. In comparison, acarbose inhibited α-amylase by 50% at 52.1 µg/mL. The results suggest that EMFZM, in particular, has bioactive substances. Although diluted in the extract, it seemed to be more effective than acarbose.

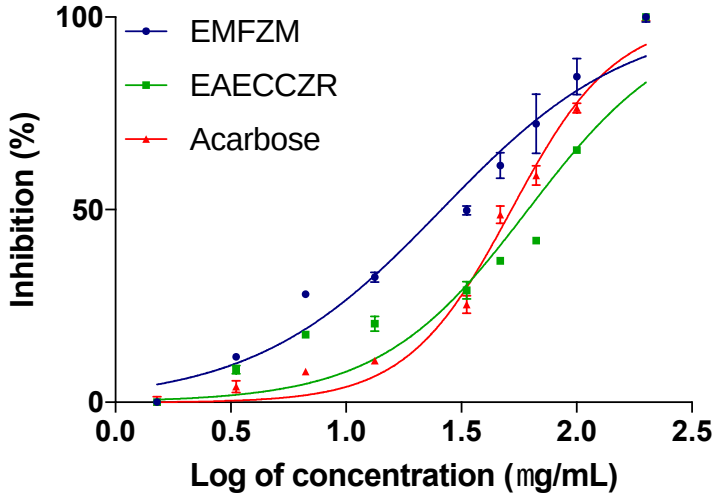


*Fig.1: Determination of $IC_{50}$ values of Zanthoxylum plant extracts on α-amylase.*

EMFZM: Methanolic extract of leaf of *Z. monogynum.* EAECCZR: Ethil Acetate extract of stem bark of *Z. rhoifolium*.

The extracts were also evaluated for their ability to inhibit α-glucosidase activity. The results showed that eleven *Zanthoxylum* extracts were able to inhibit α-glucosidase activity likewise acarbose (Table 2).

*Table 2. Inhibition of α-glicosidase by plant extracts of genus Zanthoxylum.*

| Extracts | Inhibition (%)[a] |
|---|---|
| EMCCZM | 82.2 ± 0.6 |
| EMFZM | 90.2 ± 0.3 |
| EECGCZR | 76.5 ± 1.0 |
| EECZR | 86.0 ± 0.5 |
| EHCCZR | 88.0 ± 0.1 |
| EAECCZR | 87.8 ± 1.0 |
| EECCZR | 84.4 ± 1.0 |
| EMCCZR | 99.2 ± 0.3 |
| EHFZR | 90.6 ± 0.3 |
| EAEFZR | 96.8 ± 0.3 |
| EMFZR | 81.4 ± 0.3 |
| Acarbose | 92.6 ± 0.2 |

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[a] The enzyme was preincubated with the extracts (100 µg/mL) and acarbose (100 µg/mL) for 15 min. Data were tested by ANOVA followed by Bonferroni test. EMCCZM: Methanolic extract of *Zanthoxylum monogynum* stem bark. EMFZM: Methanolic extract of *Zanthoxylum monogynum* leaves. EECGCZR: Ethanolic extract of thick stem bark *Zanthoxylum rhoifolium*. EECZR: *Zanthoxylum rhoifolium* ethanolic stem extract. EHCCZR: *Zanthoxylum rhoifolium* hexane stem bark extract. EAECCZR: Ethyl acetate extract of *Zanthoxylum rhoifolium* stem bark. EECCZR: *Zanthoxylum rhoifolium* ethanolic stem bark extract. EMCCZR: *Zanthoxylum rhoifolium* stem bark methanolic extract. EHFZR: Hexane Extract of *Zanthoxylum rhoifolium* Leaves. EAEFZR: Ethyl acetate extract of *Zanthoxylum rhoifolium* leaves. EMFZR: Methanolic extract of *Zanthoxylum rhoifolium* leaves.

In turn, methanol EMCCZM and EMFZM extracts of *Z. monogynum* had a similar inhibition potential over α-glucosidase. In the case of *Z. rhoifolium*, the thick stem bark extract (EECGCZR) showed an inhibition of 76.5%. Although lower than the values found for the other extracts, it is still considerable. In turn, the ethanolic stem extract (EECZR) presented an inhibition of 86.1%. Among the crude extracts originating from the stem bark of this plant, the methanolic extract EMCCZR was the one that stood out by inhibiting 99.2% of enzyme activity, while the other extracts EHCCZR, EAECCZR, EECCZR showed similar inhibition on α- glucosidase (88.0, 87.8 and 84.4%, respectively). Finally, the extracts obtained from the leaves were evaluated. Hexanic extract (EAEFZR), methanolic extract (EHFZR), ethyl acetate extract (EMFZR) reduced α-glucosidase activity by 96.8, 90.6 and 81.4%, respectively. These results suggest that compounds capable of inhibiting α-glucosidase are equally distributed in the different parts of both plants.

After screening, it was found that all extracts were able to inhibit α-glucosidase by more than 50% and, therefore, the $IC_{50}$ value was determined for all of them and the results are presented in Table 3.

*Table 3. IC50 determination of Zanthoxylum extracts on α-glucosidase.*

| **Extracts** | **$IC_{50}$ (µg/mL)** |
|---|---|
| EMCCZM | 24.1 ± 0.5 |
| EMFZM | 24.7 ± 0.8 |
| EECGCZR | 24.5 ± 0.2 |
| EECZR | 25.1 ± 0.4 |
| EHCCZR | 28.6 ± 0.7 |
| EAECCZR | 22.4 ± 1.5 |
| EECCZR | 26.5 ± 0.4 |
| EMCCZR | 25.1 ± 1.3 |
| EHFZR | 22.2 ± 0.2 |
| EAEFZR | 21.6 ± 1.4 |
| EMFZR | 23.5 ± 2.2 |
| Acarbose | 19.5 ± 0.2 |

Data were tested by ANOVA followed by Bonferroni test. EMCCZM: Methanolic extract of *Zanthoxylum monogynum* stem bark. EMFZM: Methanolic extract of *Zanthoxylum monogynum* leaves. EECGCZR: Ethanolic extract of thick stem bark *Zanthoxylum rhoifolium*. EECZR: *Zanthoxylum rhoifolium* ethanolic stem extract. EHCCZR: *Zanthoxylum rhoifolium* hexane stem bark extract. EAECCZR: Ethyl acetate extract of *Zanthoxylum rhoifolium* stem bark. EECCZR: *Zanthoxylum rhoifolium* ethanolic stem bark extract. EMCCZR: *Zanthoxylum rhoifolium* stem bark methanolic extract. EHFZR: Hexane Extract of *Zanthoxylum rhoifolium* Leaves. EAEFZR: Ethyl acetate extract of *Zanthoxylum rhoifolium* leaves. EMFZR: Methanolic extract of *Zanthoxylum rhoifolium* leaves.

The activity of α-glucosidase was reduced by EMCCZM, EMFZM, EECGCZR, EECZR, EHCCZR, EAECCZR, EECCZR, EHFZR, EAEFZR, EMFZR extracts with $IC_{50}$ values of 24.1, 24.7, 24.5, 25.1, 28.6, 22.4, 26.5, 25.1, 22.2, 21.6 and 23.5 µg/mL, respectively. These values did not differ statistically from each other or from acarbose

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inhibitor, whose $IC_{50}$ was in the range of 19.5 µg/mL (Figure 2). These finding indicates that the molecule responsible for enzyme inhibition is dispersed in all organs of the plant and the application of bioguided fractionation techniques may help in the identification and isolation of this substance.

For the species *Z. monogynum*, only one phytochemical study on stem bark is available in the literature so far, where the presence of alkaloids, triterpenes and steroids was identified (Moura et al., 2012). However, there are no publications on the leaves of the species. Likewise, there are no data in the literature about the chemical composition of the thick stem bark of *Z. rhoifolium*. As for the stem, the phytochemical analysis identified twelve benzophenanthridine alkaloids, six alkaloids with furoquinoline nucleus, one pentacyclic triterpene, lupeol and one steroid, β-sitosterol (Krause et al., 2013; Krause, 2013).

The stem bark extracts of this study were produced in solvents of different polarity and the literature reports the predominant presence of flavonoids in this part of the plant (Santos et al., 2013; Turnes et al., 2014). The phytochemical analysis of *Z. rhoifolium* leaves also identified alkaloids with benzophenanthridine nucleus, alkaloids, triterpene, steroids and flavonoids (Tavares et al., 2014; Christofoli et al., 2015). Another systematic study of *Z. rhoifolium* leaves revealed the presence of flavonoids in the ethyl acetate fraction and steroids and triterpenoids in the hexane fraction (Krause, 2013). Although not evaluated in this study, steroids and triterpenoids may be responsible for the effects observed in this study. In general, the literature associates the inhibition of α-amylase and α-glucosidase with the most diverse secondary metabolites of plants. Phenolic compounds, triterpenoids, tannins and flavonoids have been described as α-amylase inhibitors (Ponnusamy et al., 2011; López and Santos, 2015). In turn, α-glucosidase can be the target of more than 400 plant molecules, including phenols, terpenes, alkaloids, quinines, flavonoids, phenylpropanoids, steroids, among other types of compounds (Yin et al., 2014). Recently, an *in vivo* study conducted with the species *Z. alatum* Roxb. revealed the hypoglycemic potential of the plant, which was associated with the presence of phenolics (Suman, 2014).

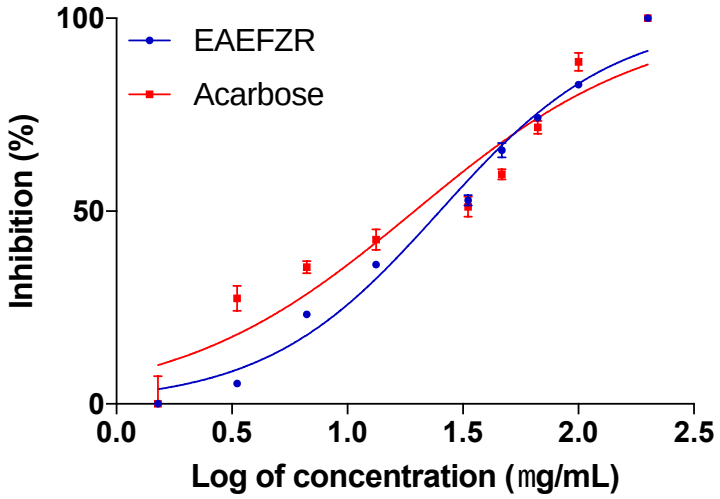


*Fig.2:* IC50 determination extracted EAEFZR on α-glucosidase.
EAEFZR: Ethyl acetate extract of *Zanthoxylum rhoifolium* leaves

.Thus, as phenolic derivatives are described in the literature as important inhibitors of α-amylase and α-glucosidase, the concentration of these compounds was determined in plant extracts by the Folin-Ciocalteau method (de Lima et al., 2014; Marques et al., 2014). The results are shown in the Table 4 as equivalent micrograms of gallic acid per milligram of extracts.

The extracts with the highest phenolic concentration were EMCCZM, EMFZM and EMCCZR (126.1, 155.2 and 145.8 µg EAG/mg, respectively). However, only EMFZM significantly inhibited α-amylase ($IC_{50}$ = 26 µg/mL), and this excludes any correlation between the concentration of these compounds and the observed effect ($p > 0.05$). In the case of α-glucosidase, all extracts inhibited its enzymatic activity in a similar manner and, therefore, there was no correlation with the presence of phenolics ($p > 0.05$). These results support the idea that inhibition of the enzymes α-amylase and α-glucosidase by *Zanthoxylum* extracts stems from metabolites other than phenolic compounds.

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Crude extracts are complex due to the diversity of secondary metabolites, many of which are not yet known. Therefore, for a better characterization of the inhibitory effect, it is necessary to fractionate the extracts in an attempt to isolate bioactive substances, as well as to determine their $IC_{50}$. It is emphasized that there is an ongoing strategic search for substances that have a greater inhibition over α-glucosidase and with a moderate effect on α-amylase in order to reduce the side effects produced by unfermented carbohydrates (Kim et al., 2011).

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